



Steven M. Ruben
Appl. No. 10/662,429

Department Molecular Biology
Subject Enzymology
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 National Brand 43-648

Computation Notebook
Dennison Stationery Products Co., Framingham, MA 01701



0 73333 43648 8

75 Sheets
11 1/4" x 9 1/2"
4x4 Quad.


BEST AVAILABLE COPY

Ruben EXHIBIT #61

Department Botany Biology
Subject Plant
Name Guo Y. G. / Y. II
Address 1-11

 43-648

Computation Notebook
Dennison Stationery Products Co., Framingham, MA 01701

 75 Sheets
11 1/4" x 9 1/2"
4x4 Quad.

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Ruben EXHIBIT 2061
Ruben v. Wiley et al.
Interference No. 105,077
RX 2061

8/21/95 Two New TNF: HPDD012 TNF epsilon
HCTBT71 TNF delta

there are differences between the two clones maybe splice variant

Oligo capture cloned full length Named HCTBT71S09

design oligos for seq Rpo2 CTCCAGCTTGGAAAGACCA
Rpo3 TTCTGCATGCCACACCTCTC
Fro6 GAACA GAAGC AAGATATCCG

design oligos for δ -specific [intron]?

design oligos for construct into pDEF70

TNF δ -SPH1: CGCGCATGCAAGGATCAAGGAGCC

TNF δ -HindIII: CGCAAGCTTACAAATCAAGTTTCAAC

TNF δ -specific oligo is given to L. Xing for Northern

8/28/95 PCR to generate insert DNA for following constructs

1. TNF α SphI + HindIII \rightarrow pOE70
2. TNF β SphI + HindIII \rightarrow pOE70
3. TNF γ d39 NcoI + HindIII \rightarrow pOE60
4. TNF γ FL BamHI + BamHI 3' \rightarrow N346
5. TNF γ FL BamHI + BamHI 3' \rightarrow New CHO vector

10 λ 10X PCR bf BM-

10 λ 2mm dNTP- CBB

oligos

0.5 μ l PWO polymerase

100 μ l

95 $^{\circ}$ C 2' 30[95 $^{\circ}$ C 1' 55 $^{\circ}$ C 1' 72 $^{\circ}$ C 1'] 75 $^{\circ}$ C 5 min

PCR \rightarrow low melting gel (Nuscreen)

4 PCR for CHO clone did not work
repeat PCR

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DATE 08/31/95

9/4/95 PCR to produce BamHI fragment for TNFR

Φ extract 2x

info tag

cells est

extract ppt



I cut - check 12 on gel

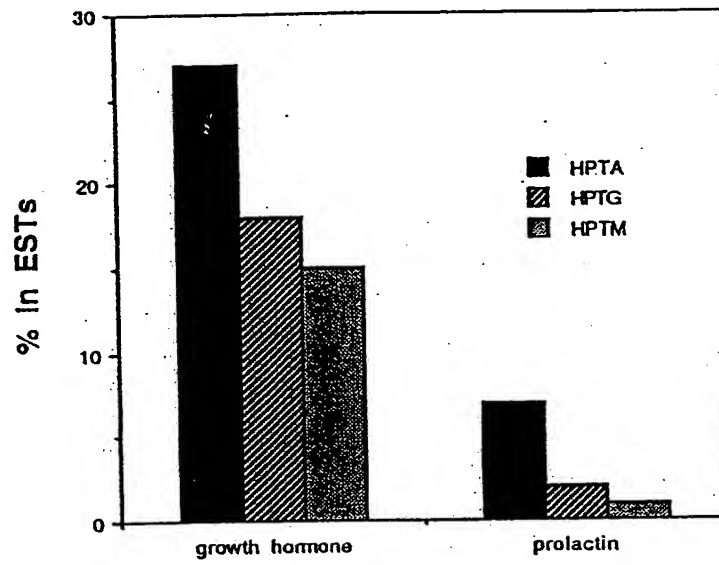
give to Lily for clone into

CHO vectors

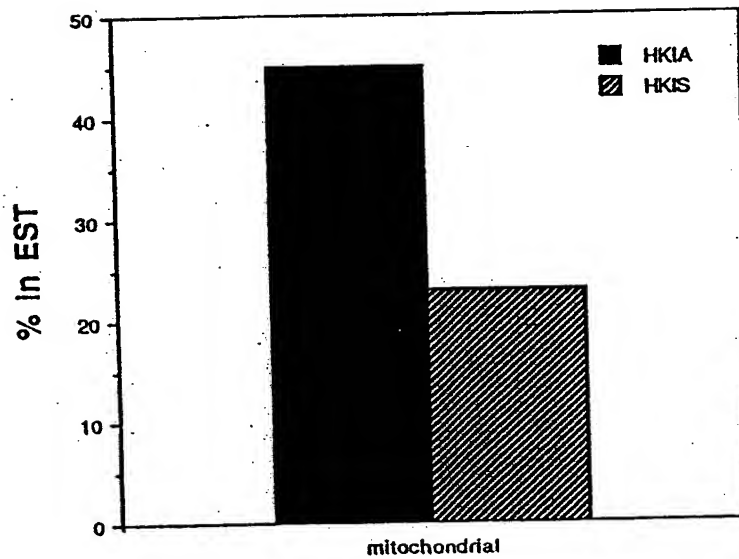
9/6/95 Summary on subtraction using biotinylated drivers generated by PCR using b2a.c4 dcrp

	Driver	PRL	G4	mito
HPTA	—	6.5%	21%	—
HPTG	G4 PL	1.6%	18%	—
HPTM	G4 PL	1.2%	15%	—
HPDB	—			34%
HSDS	mito			28%
HKIA	—			45%
HKIS	mito			23%

Subtraction result



Subtraction



9/6/95 modify the approach:

① using regulator dnap to generate ss DNA

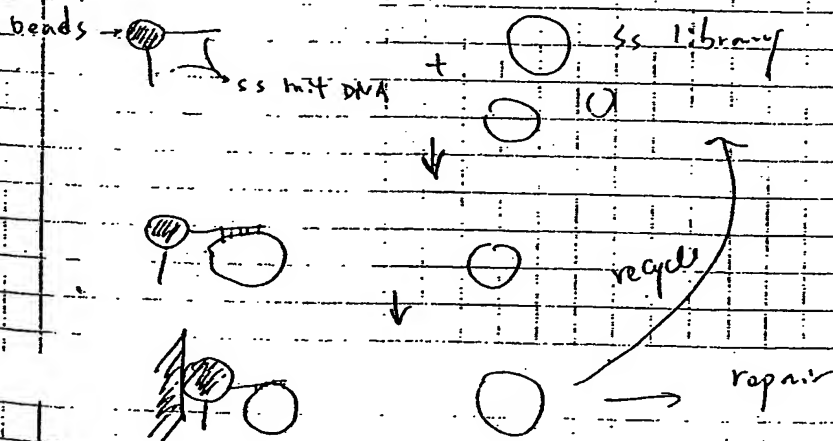
② photo biotin DNA

this is proven to be efficient

③ Try both long hybridization at 42°C and
and short hybridization at RT 1hr

④ remove biotin by extract add one more step showed
no difference by using magnetic beads

9/15 Discussion w/ Fouad idea



17 120412-72

NORTHERN BLOT DATA SHEET

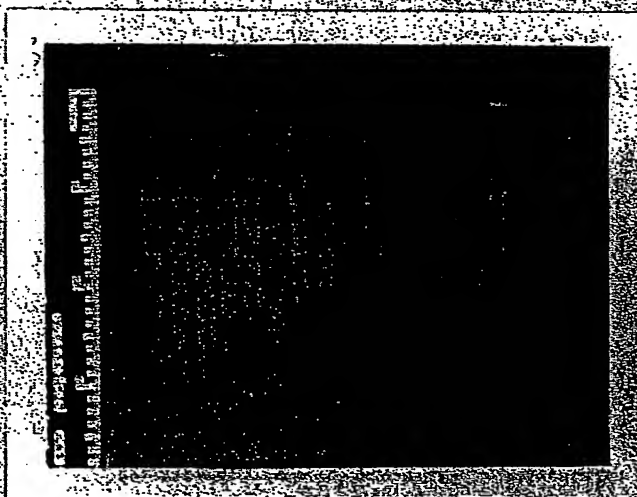
OPERATOR: Lily Xing

DATE 9/1/94

LANE #	NAME	RNA (μ l/10 μ g)	Note
1	Brain	20 μ l	
2	Kidney	"	
3	small intestine	"	
4	testis	"	
5	pancreas	"	
6	prostate	"	
7	Heart	"	
8	Liver	"	
9	lung	"	
10	thymus	"	
11	spleen	"	
12	placenta	"	
13	Colon	"	
14	ovary	"	
15	leukocyte	"	
16	muscle	"	
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20			

NOTE:

#3 gel



NORTHERN BLOT DATA SHEET

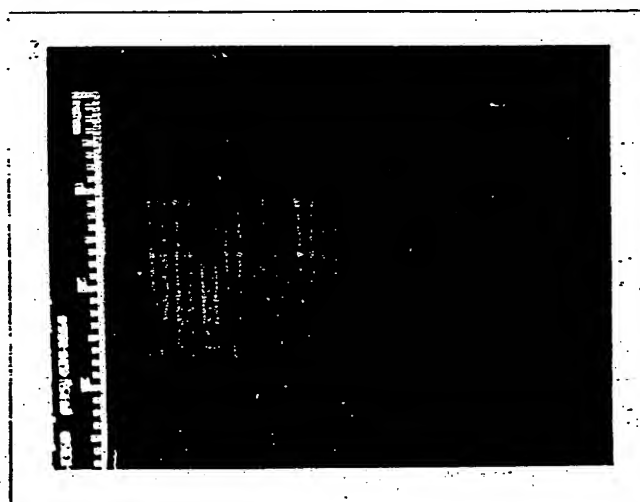
OPERATOR: Lily Xing

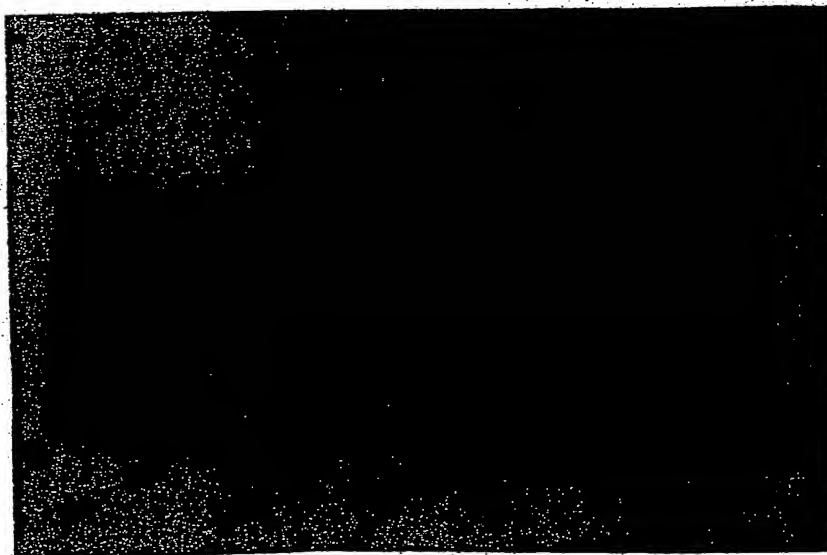
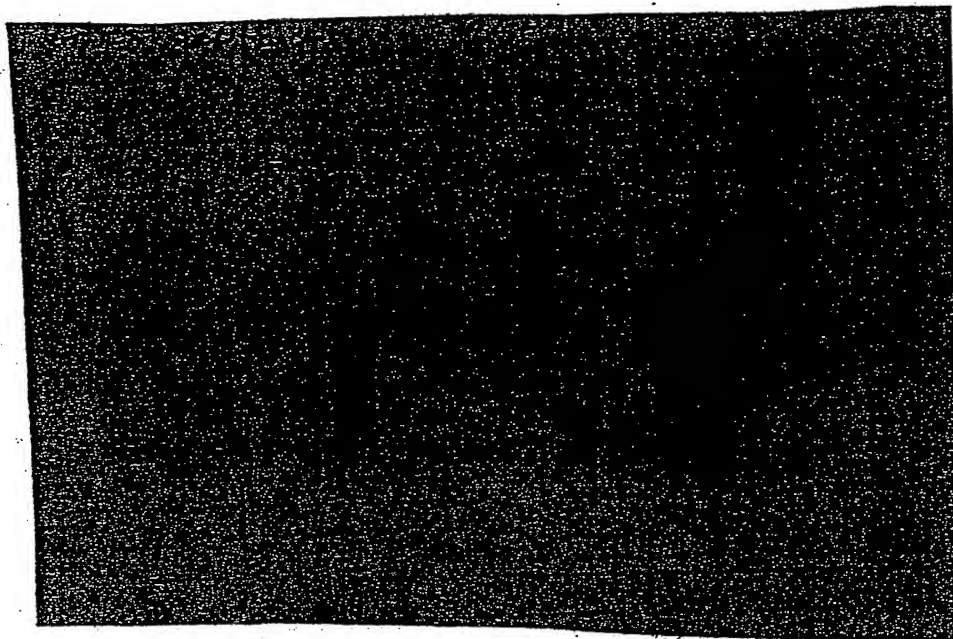
DATE 9/1/94

LANE #	NAME	RNA (μ l/10 μ g)	Note
1	Brain	20 μ g	
2	Kidney	"	
3	Small intestine	"	
4	Testis	"	
5	Pancreas	"	
6	Prostate	"	
7	Heart	"	
8	Liver	"	
9	Lung	"	
10	Thymus	"	
11	Spleen	"	
12	Placenta	"	
13	Colon	"	
14	Ovary	"	
15	Leukocytes	"	
16	Muscle	"	
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NOTE:

#3 gel





9/18/95 Design oligos for SOD4 to determine which ATG is the real ATG

3' oligos BAA TTA ACC TCA CTA AAA GGG CCA TCA TG G G CAG CG G C C A A
T₃ - SOD ATG

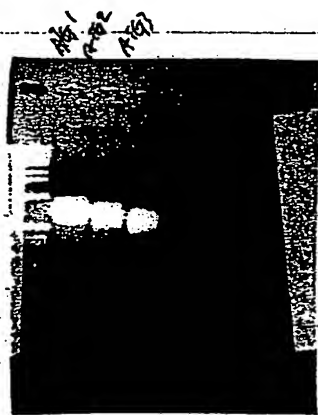
SOD ATG₂ _____ ATG G T C T T G G T A C A C

SOD ATG₃ _____ A T G A C C T G T C A G A G C

PCR to generate PCR product for T₃

3' olig SOD END

CGTCTAGAGGTCCTGCTCAAA GGTGGG



SOD4 T₃ + T₇ PCR



9/19 Subtraction Result

Sequence of two new HPT subtracted library come back
in HPTX which is long hyb (0.12 nt 4°C)

GH reduced to 10% protection to 0%

HPTX — short hyb is not as good

GH → 15% protection 3%

→ try hybridization with 5% PEG

9/20/95 TNT

promega kit

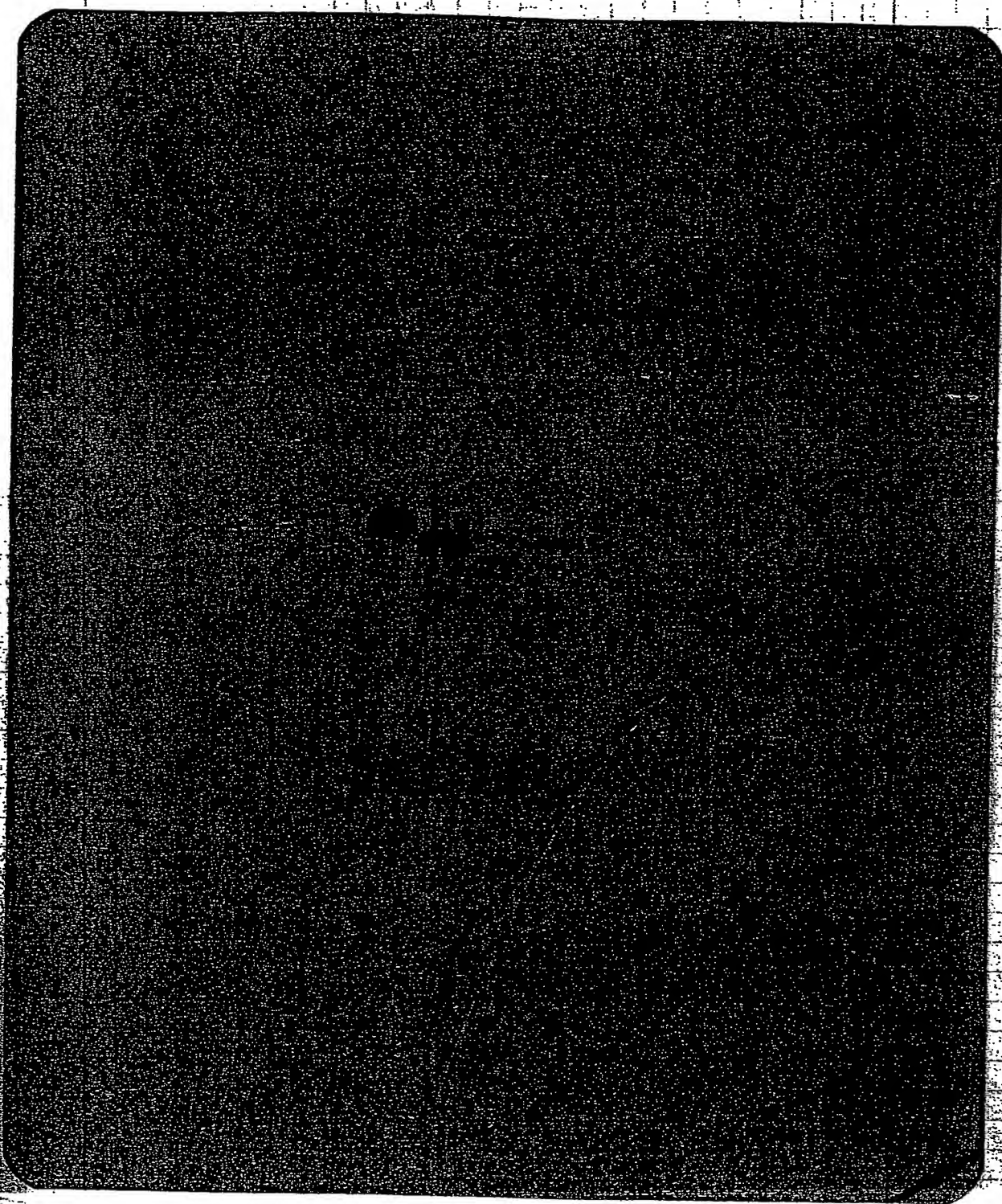
DNA: pBluescript sk
500 full length in pBluescript
PCR product from 8161
8162
8163

Tg - Cs616 in PAX

make a premix

{ 12.5 ml Rabbit Reticulocyte lysate
0.1 ml bt
0.5 ml T₂ or T₃ RNA polymerase
0.5 ml Am¹²⁵ Acl - met
2 ml 35S - met
0.5 λ RNA inhibitor from Bolygen
3 λ DNA
8 λ ddH₂O

-30°C 1 hr



9/25 repeat TNT

10x reaction
 2nd PCR product similar
 amount

Full length	SDS		
ATG1	-	1	27 Kd
ATG2	-	2	24 Kd
ATG3	-	3	21 Kd

30°C 1 hr

add 10x loading dye boil for 2 min
 Load 10x on 10% SDS gel

This experiment
 indicates there is
 another ATG upstream
 maybe the next ATG
 check seq. if
 there is another
 19 AA upstream

This will make
 the protein

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DATE

09/27/93

10/4/95

7
27 Kd
24 Kd
21 Kd

Subtraction result came back with a nice improvement
by adding 5% PEG (P200) in hybridization mix. it may be increased
hybridization efficiency

result:	before subtraction:	GH	pr
		27%	7%
	after subtraction:	5%	1%

— clone Full length TGF α by PCR HPD 1.6kb

HPD was mass excised and used to PCR using

T₁₃ Reverse + HLTB771Fp06
+ HLTB771Fp04

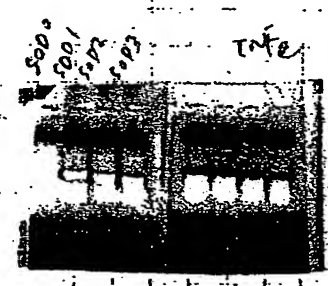
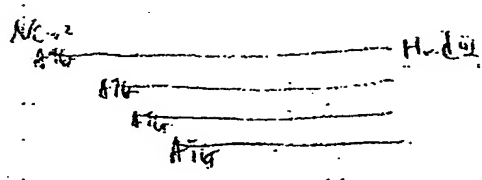
— pure band of 0.8 kb seen

— Second PCR

1) first PCR

T₃ + HLTB771Fp05
Fp06

— PCR to construct subcloning
expression vectors



— Lily will clone into 705-60

10/5/85 Gene trap

genes: TNFR p55
 TNFS
 TGFP — WWH
 Rad16 — YFW

Label oligos: ✓ TDT

TGFP 11362
11356

TNFS TNFS Rpo1

TNFR p55 cap1
+
Rpo2Rad16 - R8
+
R11

Labeling nd working

produce SS DNA by gene T EST

pCrivspat library

Leukocyt

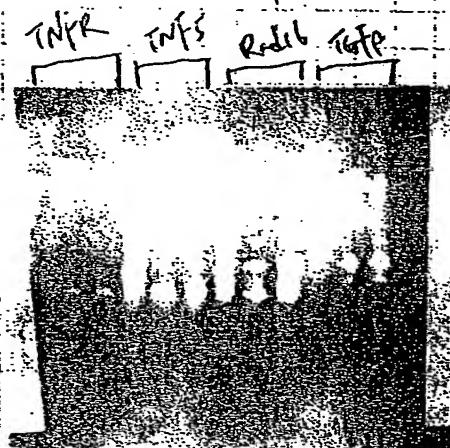
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 Gen 3638
 Gen 3641
 Gen 3644
 Gen 3647
 Gen 3650
 Gen 3653
 Gen 3656
 Gen 3659
 Gen 3662
 Gen 3665
 Gen 3668
 Gen 3

10/6/95 Repeat biotin labeling of oligos

ethanol ppt oligos final concentration 200 ng/μl

3/μg out (12 μl)

10 μl 5x TdT bfr
5 μl biotin-CTP-dep
5 μl TdT



10/9/95 Capture

add 6 μl 4x bfr Δ 95°C 1'

add 5 μl TNFR oligo to Brain

0.5 μl other oligos to Leukocyte - TNFS

Leukocyte - Rad66

WVX - TGFP

Hyb:

37°C 1 hr

treat 5A - Magnetic beads, capture, wash

repair all 10 μl

10/9/95

transfer to DH10B from BRL

plate

10 λ 100 λ

TNFRp55 —

40 colony

TNFS —

41

TGFP —

1

RAD 16 —

6

add 1ml 2x freeze buffer to remaining

10/10/95 PCR to identify positive clones

2 gene specific primers

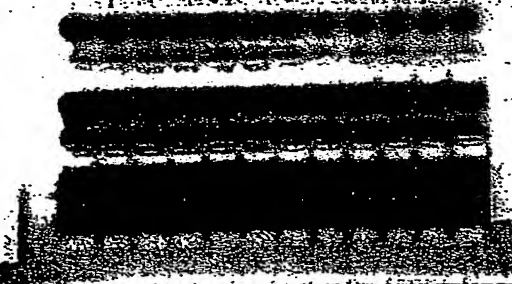
TNFS



50% clones are positive

2 gene specific primers

TNFRp55



~50% clones positive



not so colony hybrid

Show only 32/1000
hybridize

GAP + TNFS/EP1



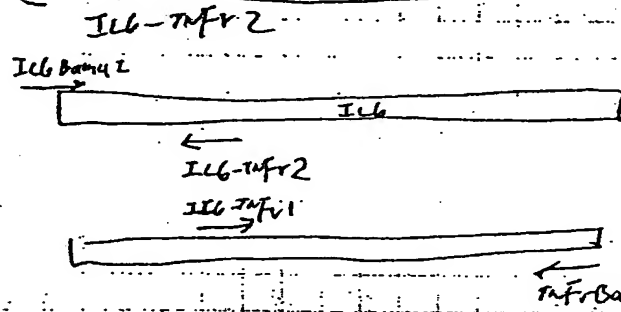
10/16/95 Fusion construct for TNF α with IL6 signal

PCR:

oligos: IL6 BamHI

C G C G G A T C C A T G A A C C A T G A A C T C C T T C T C C A C
Met

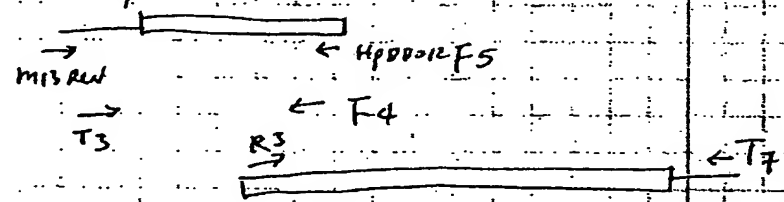
IL6-TNF α 1 IL6 TNF α
G A G G T T C C C G G T T G T G A G A C A A A C T C



50 ul reaction ss program

Fusion for Full length TNF α (HPD012)

PCR from HPD library



SUPERVISOR

DATE 10/17/95

Signature

10/23 seq analysis of 4 HTBN61 clones

HTBN61 508 — wrong clone

HTBN61 523 — wrong clone

HTBN61 502 — wrong clone

HTBN61 507 — wrong clone

design new primers for oligo capture?

have L ly in a colony hybridization

the construct of IL6/TNF α is missing kozak sequence
new oligo is made IL6's'

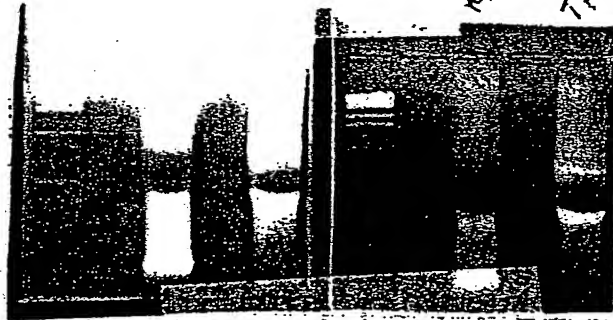
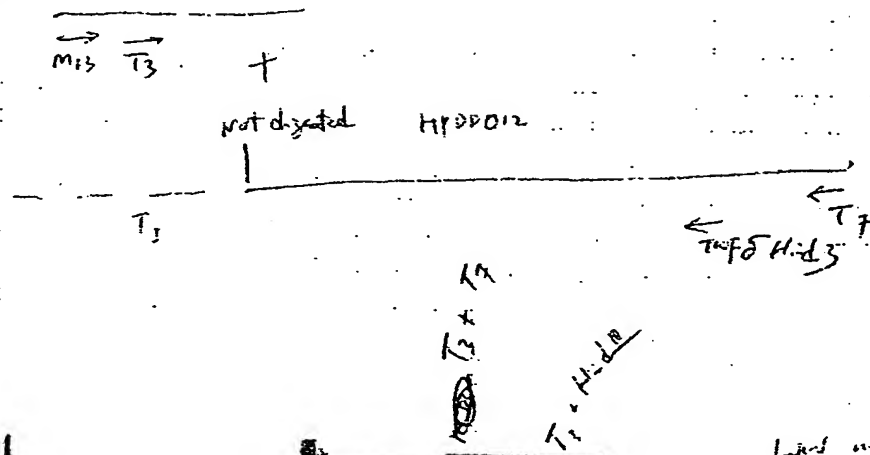
GCG GGATCC GCGGCC ATG TCC TTC TCC GC
BamHI kozak met

PCR to generate IL6/TNF α fusion

clones are made into the two CHO vectors by 2.1.1

10/26 still having problem get HpaD912 PCR fragment

PCR products



last melting gel
 Φ 2x
 CHCl₃

1 kb
 0.6 kb

digest w/ Hind3 + BamHI

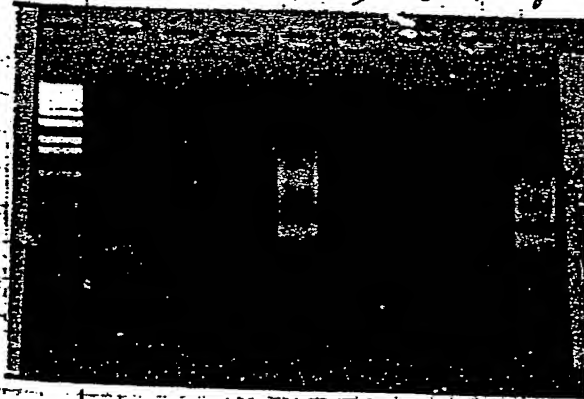
clone into pbluescript plus + lacZ

primer template

10/26

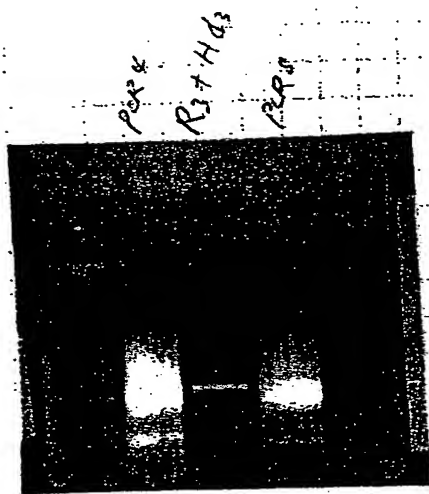
PCR products 5 μ l/50

1 2 3 4 5 6 7 8



- | | | | | |
|---|--------------|-----------|-----------|---------|
| 1 | T3 + F2 | } HpaD912 | - PCR @ - | HpaD912 |
| 2 | T7 + R3 | | | |
| 3 | T7 + F2 + R3 | | | |
| 4 | T3 + F2 | } HpaD | - PCR @ - | HpaD |
| 5 | T3 + F2 | | | |
| 6 | T7 + R3 | | | |
| 7 | T7 + F2 + R3 | - PCR @ - | HpaD | |
| 8 | T3 + F2 | | | |

10/17 low melting gel



cut out DNA

PCR 4 → 0.6 - 1.1 kb

PCR 8 -

R3 + Hd3

(PCR 7)

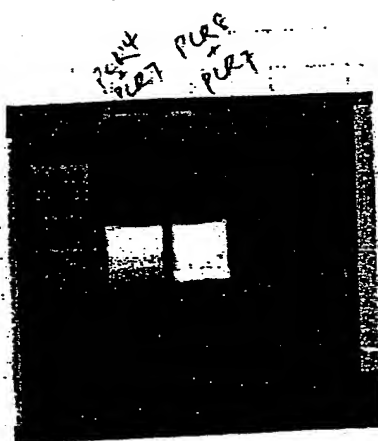
Φ 2x

cut, 1x

extract. p₁

mix PCR 4 + PCR 7
PCR 8 + PCR 7

PCR using T3 + Hd3



low melting gel purify top band

HindIII + BamHI digestion

clone into p Bluescript HindIII + BamHI digestion

PCR check → no clone found

11/7/95

5:30

flanking Tnf α Rpo7 + Rpo7

DNA

primer

PCR:

① HPD 1.6m

Rpo7 + Fpo7

② HPD 1.6m

Rpo7 + Fpo7 + Tnf α BamHI + Tnf α HindIII

③ HPD 1.6m

Tnf α BamHI + HindIII

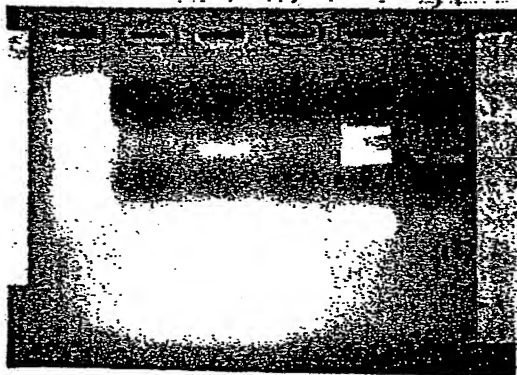
④ HLTBT71.5g

Rpo7 + Fpo7 $\xrightarrow{\text{expect}}$ 1.7 kb

⑤ HLTBT71.5g

5' BamHI + 3' HindIII $\xrightarrow{\text{expect}}$ 600 bp

1 2 3 4 5

wrong size for Tnf α products

10/10

New primer made for Tnf α BamHI

PCR

DNA

primer

① HPD 1.6m

Rpo7 + Fpo7

② HPD 1.6m

BamHI + HindIII

③ PCR ①

Rpo7 + Fpo7

④ PCR ①

BamHI + HindIII

⑤ HLTBT71.5g

Rpo7 + Fpo7

⑥ HLTBT71.5g

EcoRI + HindIII

1 2 3 4 5 6



140

11/11/95 2% can melting gel purification PUR 2 & PCR C

Φ1 Φ1000z endr exhaust ppt

digest with BamHI + H-d

also digest Vector pOE-9

Ligation on

Design oligos to test ligation for SAGE

Ligation oligo 1 GAGTCAGTTCATG CCAACGGCATG

Ligation oligo 2 CCGTTTGGCATTG AACTGACT CCATC

SUPERVISOR-

DATE-

11-20-95

Patricia Dillon

11/20/95 RACE for HTBN61 TNFRp55 homologs

The following library has the gene based on PCR using 2 gene specific primers

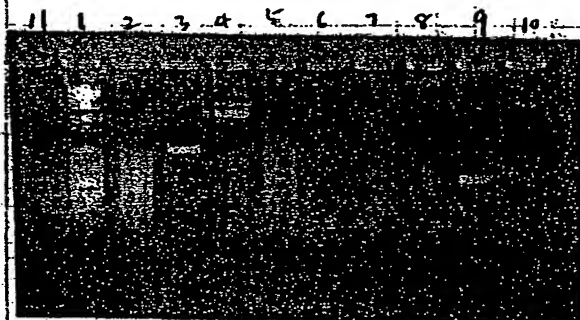
HCO HLO HFV HFB HPD HAP HTX HLE9
1 2 3 4 5 6 7 8

M13RV T3

← F3 ← F4

PCR using M13RV + F4

9 = HPD
TNFRp55 + Fp7
10 : RpoE + Fp6
11 : RpoB + 5HindIII



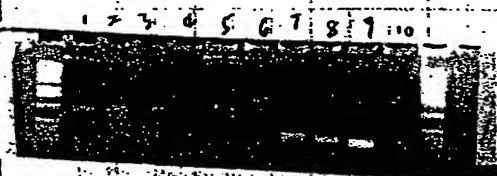
Second PCR

T3 + F3

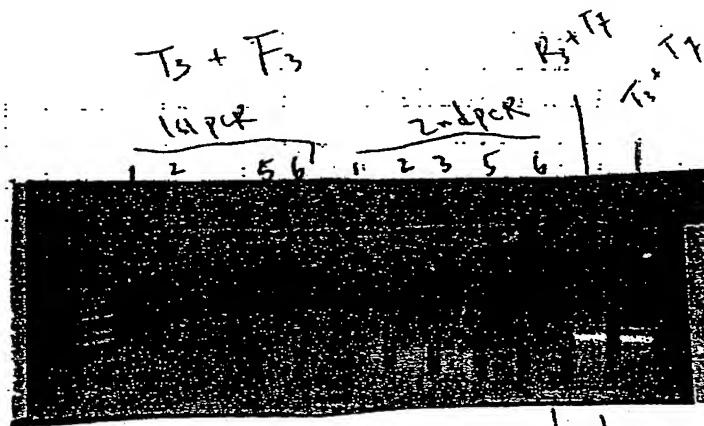
1-8 template PCR 1-8

9 HTBN61

10 HTBN61 T7 + Rpo1



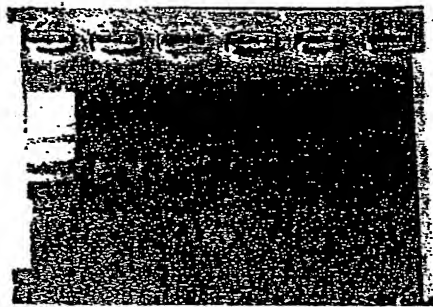
11/21/95



5λ + 10% (gel purified)

$T_3 + T_7$ PCR

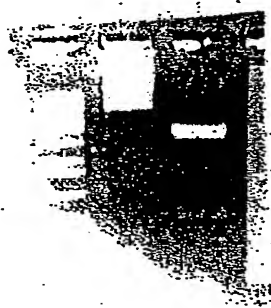
11/28



no specific products

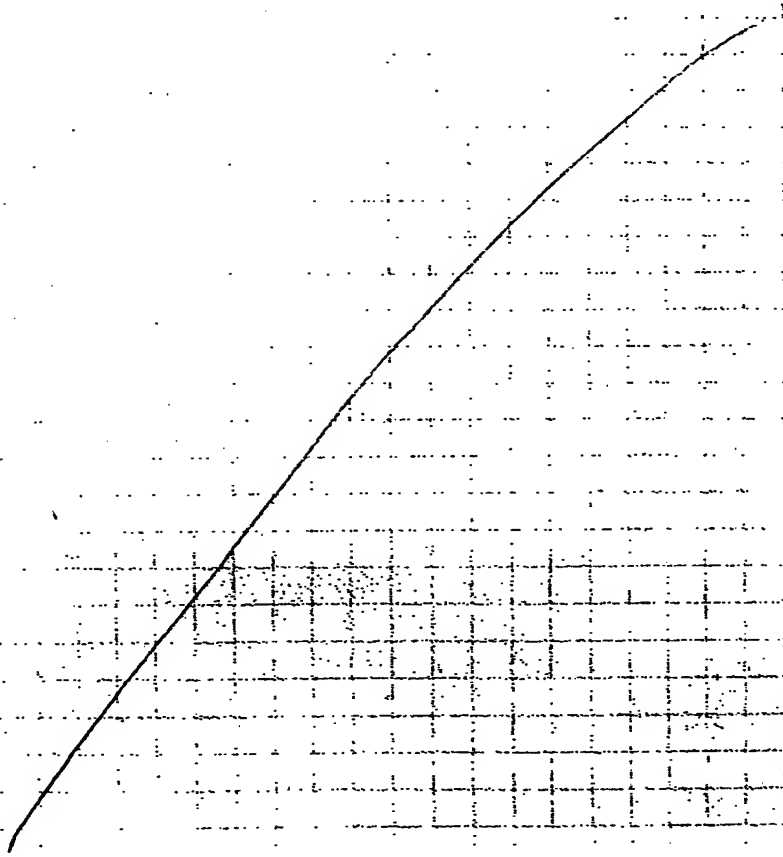
11/25 Trf 5 His tag construct

Seq verified digestion w/ Bam + Hind III



Signed by R.G. and K.R.

gave DNA to protein expression



12/6/95 prepare linkers for SAGE

large scale oligo synthesis each 7.5 mg

linker A

TTTTAATTAACCCCTCACTAAAGGGCTCGCACGGATGCATG 4368
TTAATTGGGAGTGATTCCCGAGCGTGCCTAC 4370

linker B

TTTGTGAATACGACTCACTATAGGGCAAGTCGGATGCATG 4369
CATTATGCTGAGTGATATCCCGTTCAGCCTAC 4371

12% urea acrylamide gel purify the oligos
put through C18 column

O.Y. ϕ /C18 extr ethanol ppt + equal vol 1M Tris pH 7.5
3.5 vol 100% EtOH

Concentration

Sample	abs			260.0 nm	
	260.0 nm	280.0 nm		260.0 nm	280.0 nm
4368	0.3014	0.1508	4.5 v/v	1.5797	0.6339
69	0.3548	0.2061	5.3	1.7045	0.5367
71	0.3056	0.1575	4.5	1.5468	0.6465
71	0.3139	0.2102	4.5	1.5171	0.5591
88	0.0782	0.0489	1.1	1.5531	0.6414
69	0.0709	0.0456	1.2	1.7304	0.5773
70	0.1143	0.0757	1.7	1.5114	0.6616
71	0.1609	0.0950	1.5	1.5292	0.6539

ϕ extracted

gel purified

Anneal

80 μ g (4368) + 64 μ g (4370) + 20 μ l 10x kinase buffer

80 μ g (4369) + 64 μ g (4371)

70 mM Tris-HCl

10 mM MgCl₂

1 mM DTT

add H₂O to 200 μ l

A B gel purified
A' B' ϕ 100₃/chool 11T

65°C → cool to 4°C put heat block on bench

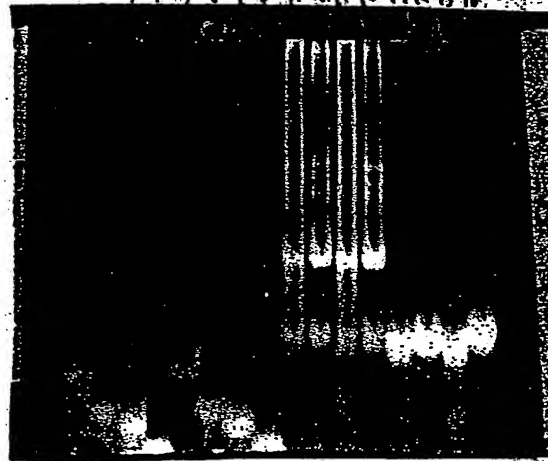
add 1 λ 100 mm A-T-P
1 λ 100 mm ddA-T-P
2 λ P-N-K
2 λ Klenow

37°C 1 hr

take 2 λ each ligate in 10 λ 2 λ S-L BRC ligation buffer
1 λ NEB high conc ligation

RT 2-3 hrs

12% native agarose gel

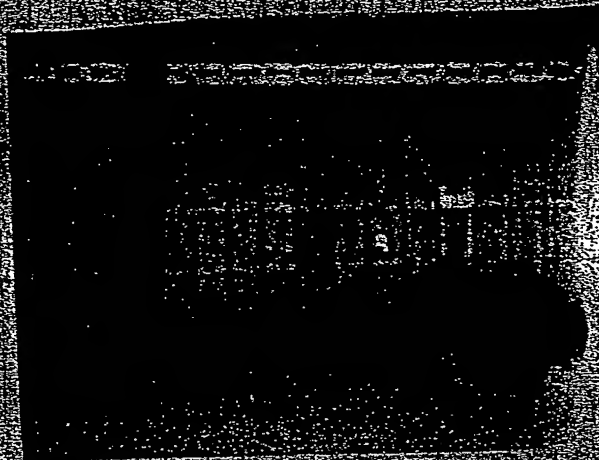


1	4368	} ϕ 100 ₃ chool 11T
2	4370	
3	4369	
4	4371	
5	4368	} gel purified
6	4370	
7	4369	
8	4371	
9	Linker A	} Self ligation
10	Linker A'	
11	B	
12	B'	
13	Linker A	
14	A'	
15	B	
16	B'	

12/12/95 Northern form HTTBal61 TAFR

1	2	3	4	5	6	7	8	9	10	11	12	13
1. 1st	2. 2nd	3. 3rd	4. 4th	5. 5th	6. 6th	7. 7th	8. 8th	9. 9th	10. 10th	11. 11th	12. 12th	13. 13th

14. 14th



12/11/95 SAGE
Linkers A and Linkers B

13
Gallballe
new

	12/11/95	12/11/95	12/11/95	12/11/95	12/11/95
12/11/95	12/11/95	12/11/95	12/11/95	12/11/95	12/11/95
12/11/95	12/11/95	12/11/95	12/11/95	12/11/95	12/11/95
12/11/95	12/11/95	12/11/95	12/11/95	12/11/95	12/11/95
12/11/95	12/11/95	12/11/95	12/11/95	12/11/95	12/11/95

HBC library plasmid DNA digest w/ XhoI
total 7 μ g



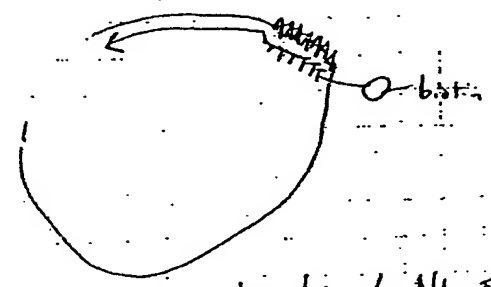
take $\frac{1}{2}$ (3 μ g)

\downarrow 96°C 5 min

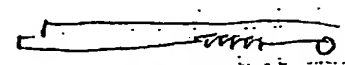
add 0.5 μ g biotinylated oligo dT (LNES)

\downarrow 37°C

Klenow 1 μ l 100 mM dNTP
30 min



\downarrow digest w/ NlaIII

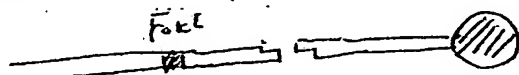


\downarrow ligation in 20 μ l Pfu buffer
RT 3 hr

Linker A
0.2 μ g

Linker B
1.2 μ g

12/12 prewash streptavidin magnetic beads
 TE wash 3X
 resuspend in 30 λ TE add ligation
 bind 1 hr at RT with occasional
 wash w/ TE/1m NaCl 2X transfer to new tube
 wash 2X more
 digestion with FokI at 37°C



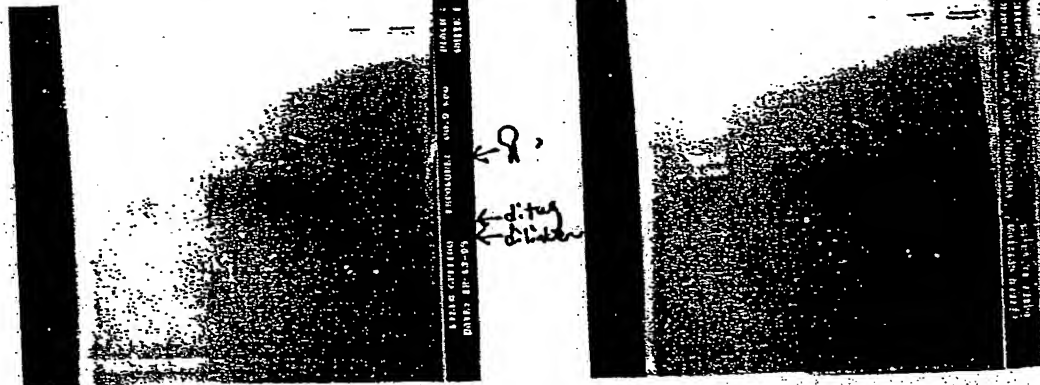
Φ /Cts et al. pTV T4 DNA pol/uvase
 +
 1 λ 100mM dTP
 RT 30'

↓ Ligation
 8°C

mix A + B

↓ PCR T3 + T7 66 program
 0.1 1. 4 λ ligation as template (30 cycles)

0.1 1. 4



12/12 prewash streptavidin magnetic beads

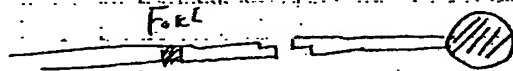
TE wash 3X

resuspend in 30 λ TE add ligation

bind 1 hr at RT mix occasionally

wash w/ TE/1m NaCl 2X, transfer to new tube
wash 2X more

digestion with FokI at 37°C



Φ /Cts shad_{pt} T4 DNA pol_y nuclease
+ 1 λ 100mM dATP

RT 30'

↓ Ligation

mix A + B

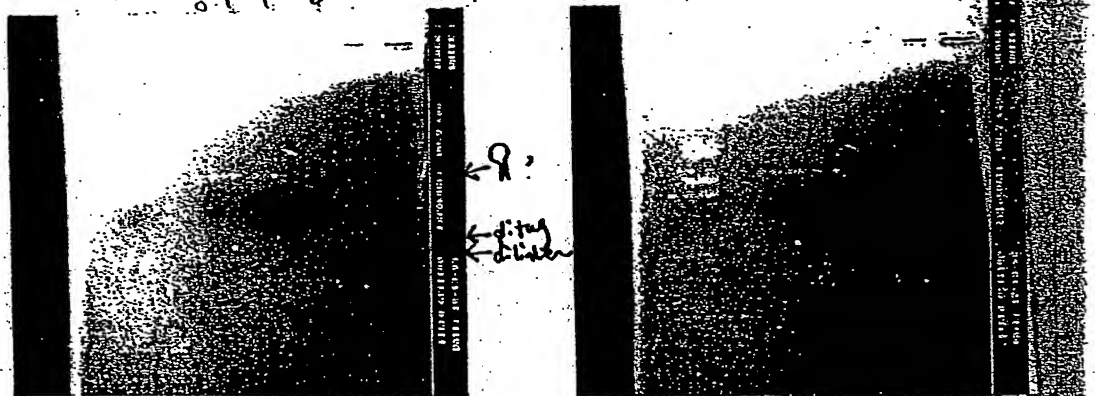
8°C

o/n

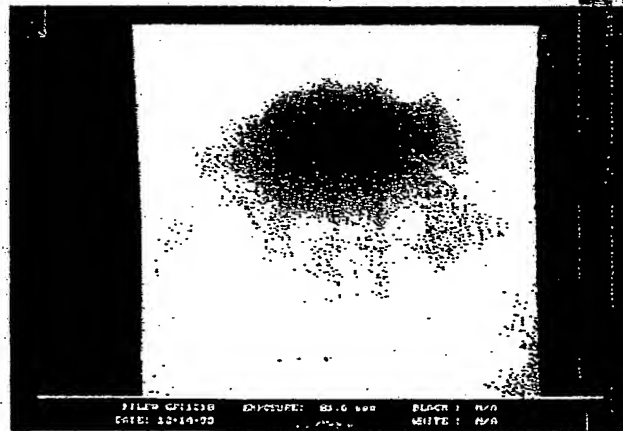
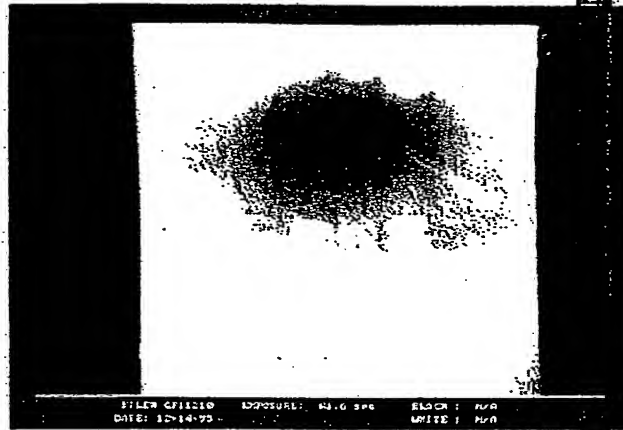
↓ PCR T3 + T7 66 program

o.l. 1. 4 λ ligation as template (30 cycles)

o.l. 1. 4



5c



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DATE: 12-14-73 WHITE: N/A

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